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## **-Deuteration of N-nitrosoethylmethylamine causes a shift in DNA methylation from rat liver to esophagus**

von Hofe, Eric ; Schmerold, Ivo ; Nims, Raymond W ; Keefer, Larry K ; Reist, Elmer J ; Kleihues, Paul

**Abstract:** While N-nitrosoethylmethylamine (NEMA) is carcinogenic primarily for the liver, its -trideuterated derivative, N-nitroso([2-D3]ethyl)methylamine (NEMA-d3), also produces a high incidence of tumors in the esophagus. To determine whether this shift in organ specificity is associated with an altered pattern of DNA alkylation, [methyl-14C]- and [1-ethyl-14C]-labeled NEMA-d3 were administered to adult male Fischer 344 rats as a single i.p. dose (0.05 mmol/kg; 4 h survival). Levels of methylated and ethylated purines in the DNA of various organs were determined by radiochromatography on Sephasorb-HP columns. When compared to previous data using undeuterated NEMA, 7-niethylguanine levels were found to be reduced by 30% in liver and kidney, but were 160% greater in esophagus. This resulted in a decrease in the 7-methylguanine ratio for liver/esophagus from 109 to 29. O6-Methylguanine was diminished in liver and kidney, but levels in lung and esophagus were too low for quantitative detection. Similarly, deuteration led to an 18% decrease of 7-ethylguanine in hepatic DNA. The observed increase in esophageal DNA methylation correlates with the increased carcinogenicity of NEMA-d3 relative to undeuterated NEMA in that organ. Since pharmacokinetic studies have shown that -trideuteration of NEMA does not alter its bioavailability, the data suggest that the observed shift in target organ results from isotopically-induced changes in the balance among competing metabolic pathways in different rat tissues

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# $\beta$ -Deuteration of *N*-nitrosoethylmethylamine causes a shift in DNA methylation from rat liver to esophagus

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While *N*-nitrosoethylmethylamine (NEMA) is carcinogenic primarily for the liver, its  $\beta$ -trideuterated derivative, *N*-nitroso([2-*D*<sub>3</sub>)ethyl)methylamine (NEMA-*d*<sub>3</sub>), also produces a high incidence of tumors in the esophagus. To determine whether this shift in organ specificity is associated with an altered pattern of DNA alkylation, [methyl-<sup>14</sup>C]- and [1-ethyl-<sup>14</sup>C]-labeled NEMA-*d*<sub>3</sub> were administered to adult male Fischer 344 rats as a single i.p. dose (0.05 mmol/kg; 4 h survival). Levels of methylated and ethylated purines in the DNA of various organs were determined by radiochromatography on Sephasorb-HP columns. When compared to previous data using undeuterated NEMA, 7-methylguanine levels were found to be reduced by ~30% in liver and kidney, but were 160% greater in esophagus. This resulted in a decrease in the 7-methylguanine ratio for liver/esophagus from 109 to 29. *O*<sup>6</sup>-Methylguanine was diminished in liver and kidney, but levels in lung and esophagus were too low for quantitative detection. Similarly, deuteration led to an 18% decrease of 7-ethylguanine in hepatic DNA. The observed increase in esophageal DNA methylation correlates with the increased carcinogenicity of NEMA-*d*<sub>3</sub> relative to undeuterated NEMA in that organ. Since pharmacokinetic studies have shown that  $\beta$ -trideuteration of NEMA does not alter its bioavailability, the data suggest that the observed shift in target organ results from isotopically-induced changes in the balance among competing metabolic pathways in different rat tissues.

## Introduction

*N*-Nitrosoethylmethylamine (NEMA\*) is a powerful liver carcinogen and of particular interest in mechanistic studies on the organ specificity of nitrosamines since it does not follow the general principle established by Druckrey and coworkers (1) that asymmetric nitrosamines produce predominantly esophageal tumors. Lijinsky *et al.* (2,3) have shown that deuteration of the  $\beta$ -carbon atom of NEMA leads to induction of esophageal carcinomas in addition to liver tumors. A possible explanation for this isotope effect on organ specificity is that the greater effective strength of the C–D bond (relative to the C–H bond) slows metabolic oxidation at the isotopically substituted

$\beta$ -position, shifting metabolism toward increasing  $\alpha$ -hydroxylation of the ethyl group and causing correspondingly more production of the powerfully alkylating methyldiazonium ion. Since rat esophagus appears to be particularly vulnerable to the carcinogenic effects of this methylating agent (4), the increased potency of NEMA-*d*<sub>3</sub> relative to NEMA in that organ would be explained if the extent of this deuterium-induced metabolic switching were sufficient to cause increased DNA methylation in the esophagus. We have previously shown that NEMA methylates, ethylates and 2-hydroxyethylates DNA of various organs in the rat (4–6). To test whether  $\beta$ -deuteration does cause a shift in the profile of DNA adducts, we have now measured the extent of DNA alkylation in various target and nontarget organs of adult male Fischer 344 rats following exposure to single doses of *N*-nitroso([2-*D*<sub>3</sub>)ethyl)methylamine (NEMA-*d*<sub>3</sub>) <sup>14</sup>C-labeled in either the *N*-methyl or 1-ethyl position.

## Materials and methods

### Animals

Adult male Fischer 344 rats (120–150 g) were purchased from Charles River Wiga GmbH, Sulzfeld, FRG. A standard laboratory diet and water were given *ad libitum*.

### Chemicals

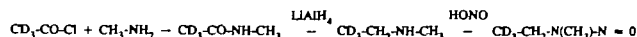
[1-Ethyl-<sup>14</sup>C]-labeled NEMA-*d*<sub>3</sub> was prepared as follows. Barium [<sup>14</sup>C]carbonate (52.1 mCi/mmol, 2.04 mmol) was treated with 10 ml of concentrated sulfuric acid and the liberated <sup>14</sup>CO<sub>2</sub> was condensed into a previously evacuated two-neck flask cooled in liquid nitrogen. Anhydrous diethyl ether (5 ml) followed by 4.49 ml of 0.454 M [*D*<sub>3</sub>]methyl magnesium iodide (2.04 mmol) in ether were injected through a rubber septum onto the frozen <sup>14</sup>CO<sub>2</sub>. The resulting mixture was stirred for 1 h at –78°C and 10 min at room temperature, then evaporated *in vacuo*. The residual, doubly-labeled acetate salt was treated with 10 ml of dichloromethane and 0.35 ml of 6 M hydrochloric acid. Water was removed from the resulting labeled acetic acid solution by filtration through anhydrous magnesium sulfate, rinsing with 15 ml of dichloromethane. The filtrate was mixed with 0.187 ml (2.2 mmol) of oxalyl chloride and refluxed for 2.5 h in a flask fitted with a –78°C cold-finger condenser. The reaction mixture was cooled to –78°C and a slow stream of methylamine was bubbled through over a 30-min period until the solution was basic to litmus. The resulting mixture was stirred at 25°C for 30 min, filtered through Celite, and evaporated to dryness. The residual 602 mg of crude, doubly-labeled *N*-methylacetamide was placed on a column of 5 g of silica gel (70–230 mesh) packed in 95:5 ether:methanol and eluted with 60 ml of the same solvent mixture followed by 45 ml of 90:10 ether:methanol to yield 68 mCi of the amide on evaporation of the eluate. The labeled amide (1.38 mmol) was taken up in 2 ml of dry tetrahydrofuran, added to a suspension of 134 mg (3.53 mmol) of lithium aluminum hydride in tetrahydrofuran, refluxed for 1 h, and cooled during the very slow addition of 4 ml of 80:20 tetrahydrofuran:water followed by 4 ml of 5 M sodium hydroxide. The resulting mixture was distilled into a receiver containing 1 ml of 3 M hydrochloric acid at 0°C, whereupon 3 ml of water was added to the distillation flask and the distillation was continued. The distillate was rotary evaporated to remove organic solvent, mixed with 2 ml of 3 M hydrochloric acid, and cooled in an ice bath. A solution of 2.86 g (41.5 mmol) of sodium nitrite in 5 ml of water was dropped very slowly into the doubly-labeled methylethylamine hydrochloride in acid. After stirring in the ice bath until addition was complete, the mixture was stirred for 3 min in a 30°C bath and extracted with four 5-ml portions of dichloromethane. The organic extracts were dried over anhydrous magnesium sulfate, concentrated to 1 ml at 0°C on a rotary evaporator, and eluted through a column of 1 g of silica gel with dichloromethane to yield 46.6 mCi of nitrosamine (44% overall radiochemical yield). Chromatography on a Waters RadialPak C<sub>18</sub> column with 50:50 methanol:water at 0.6 ml/min eluted the nitrosamine at a retention time of 7.1 min. Radioactivity detection indicated 100% radiochemical purity, while ultraviolet

\*Abbreviations: NEMA, *N*-nitrosoethylmethylamine; NEMA-*d*<sub>3</sub>, *N*-nitroso([2-*D*<sub>3</sub>)ethyl)methylamine.

detection at 210 nm indicated 99% chemical purity. The ultraviolet spectrum in water (absorbance maximum at 227 nm, extinction coefficient of 7781 l/mol/cm) was similar to that of unlabeled NEMA. The mass spectrum was consistent with the assigned structure and indicated an isotopic purity of 96.6% deuterium at the 2-position.

[Methyl- $^{14}\text{C}$ ]-labeled NEMA- $\text{d}_3$  was synthesized as previously reported (7). Its ultraviolet and chromatographic properties were similar to those for the ethyl-labeled isomer described in the previous paragraph (radiochemical purity, 98%; chemical purity, 99.5%; isotopic purity at the 2-position, 95.3% deuterium).

Nonradioactive NEMA- $\text{d}_3$  was prepared by a procedure analogous to those described above, as outlined in the following equation:



It was purified by distillation at 74–75°C (34 mm Hg), showing ultraviolet maxima in water of 228 nm (7888 l/mol/cm) and 334 nm (84 l/mol/cm), and gave a high pressure liquid chromatogram indicating a chemical purity of 99.3%. The mass spectrum revealed molecular ions at  $m/z$  91, 90, 89 and 88 atomic mass units of relative intensities 90, 9, 1 and 0 respectively, pointing to an isotopic purity at the 2-position of 96.3% deuterium.

For DNA alkylation studies, the radiolabeled compounds were used at sp. act. of 10.7 and 48 mCi/mmol for [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  and [1-ethyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  respectively. For metabolism experiments, the sp. act. of [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  was adjusted to 1.83 mCi/mmol. The radiochemical purities were checked immediately before use by HPLC on an RP 18 column (Shandon, ODS Hypersil, 4.6 × 250 mm) eluted with 10% aqueous methanol (v/v) and were found to be >98%. Riatron scintillation cocktail was from Kontron AG (Zürich, Switzerland) and Lumagel SB scintillation cocktail was from Lumac/3M bv (Schaesberg, Netherlands). Sephasorb-HP was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and hydroxylapatite was from Boehringer Mannheim GmbH, FRG. All other chemicals were of analytical grade purity or higher.

#### Metabolic Cage

Two rats were given a single i.p. injection of [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  at a dose of 4.4 mg/kg (1.83 mCi/mmol) and monitored for 14 h in a metabolic cage (Jencons Metabowl, Hemel Hempstead, United Kingdom) for expiration of  $^{14}\text{CO}_2$ , which was followed by collecting aliquots from two serially-connected Nilox columns containing 1 N NaOH. Samples (0.5 ml) from the Nilox columns were counted for radioactivity after the addition of 1.5 ml of water and 8 ml of Lumagel SB (85% counting efficiency).

#### DNA alkylation by NEMA- $\text{d}_3$ in vivo

Rats were given [methyl- $^{14}\text{C}$ ]-labeled NEMA- $\text{d}_3$  (4.4 mg/kg, 10.7 mCi/mmol, eight animals) or [1-ethyl- $^{14}\text{C}$ ]-labeled NEMA- $\text{d}_3$  (4.4 mg/kg, 48 mCi/mmol, seven animals) as single i.p. doses and killed 4 h later. Organs were rapidly removed, immediately frozen in liquid nitrogen and stored at -70°C.

DNA was isolated by phenol extraction of proteins and adsorption onto hydroxylapatite as previously described (4). The DNA from 1–2 g of tissue was deproteinized in 0.1 N HCl for 20 h at 37°C. The hydrolysate was loaded onto a Sephasorb column (1 × 50 cm) and eluted with 10 mM phosphate buffer (pH 5.5) at a flow rate of 1.6 ml/min as described earlier (8). Adenine and guanine were quantified by their absorption at 260 nm and  $^{14}\text{C}$  radioactivity was determined by liquid scintillation counting (counting efficiency 85%). Concentrations of methylated and ethylated purines were calculated assuming that their sp. act. was identical to that of the nitrosamine injected. Analysis of hydroxyethylated DNA adducts was performed using Bio-Rad AG 50W-X4 chromatography as previously described (6,9).

#### Microsomal studies

To prepare the microsomes, hepatic tissue was obtained from 14 uninduced 8-week-old rats and homogenized in 0.15 M potassium chloride/0.2 M sucrose, pH 7.4. This and all following steps were performed at 4°C. Pooled homogenate was centrifuged at 9000 g for 20 min, then at 105 000 g for 75 min in an ultracentrifuge. The resulting microsomal pellets were washed twice with potassium chloride/sucrose, pooled, and resuspended in potassium chloride/sucrose solution using a glass/glass tissue grinder. Individual aliquots were stored at -70°C. The final protein concentration was determined using fluorescamine (10) to be 48 mg/ml, with bovine serum albumin used as the standard.

To follow the microsomal metabolism of NEMA and NEMA- $\text{d}_3$ , the method of Farrelly *et al.* (11) was used. Reaction mixtures contained 0–10 mM nitrosamine, 5 mM semicarbazide hydrochloride, 1 mM EDTA, 5 mM glucose-6-phosphate, 1 mM NADP, 50 mM Tris, 1.6 mg of microsomal protein, and 0.4 units of glucose-6-phosphate dehydrogenase in a total volume of 2 ml, pH 8.0, 37°C. Reactions were started by mixing the nitrosamine with the other reactants in 25-ml Erlenmeyer flasks, and were stopped after 20 min by addition of 1 ml each of zinc sulfate (5% solution) and barium hydroxide (4.5% solution). Control incubations consisted of reactions performed in the absence of nitrosamine

or with boiled microsomes. The dinitrophenylhydrazine adducts were prepared and assayed as described (11); the  $K_m$  and  $V_{max}$  values were derived from the data as previously indicated (11).

## Results

The rate of metabolism of [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  was determined by monitoring exhaled  $^{14}\text{CO}_2$  following a dose of 4.4 mg/kg. Forty-eight percent of the administered radioactivity was exhaled as  $^{14}\text{CO}_2$  over a 14-h period. The time at which half the maximal amount of  $^{14}\text{CO}_2$  was exhaled was 1.4 h. After ~8 h the levels of expired radioactivity plateaued at a value 48% of the administered dose. As this rate of metabolism was comparable to that observed for metabolism of undeuterated NEMA (5), the same survival time (4 h) was used for DNA alkylation studies.

Sephasorb-HP radiochromatography of hydrolyzed DNA revealed that  $\text{O}^6$ -methylguanine and 7-methylguanine were the major alkylated purines in all organs examined from [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$ -treated animals and that  $\text{O}^6$ -ethylguanine and 7-ethylguanine were the major radioactive peaks in chromatograms from [1-ethyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$ -treated animals. An early peak which eluted between pyrimidines (in breakthrough fraction) and 7-ethylguanine was observed in hepatic DNA from animals receiving [1-ethyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$ . Subsequent chromatography using Bio-Rad AG 50W-X4 (which is capable of resolving 3-ethyladenine and 7-hydroxyethylguanine) suggested that this peak was most likely 3-ethyladenine (data not shown). No hydroxyethylated purines could be detected using this latter chromatographic system.

The extent of DNA alkylation in liver, kidney, lung and esophagus by NEMA- $\text{d}_3$   $^{14}\text{C}$ -labeled in either the *N*-methyl or 1-ethyl position is shown in Table I. The results obtained from previous experiments with non-deuterated NEMA (5) are presented for comparison. The administration of [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  resulted in ~30% less DNA methylation in liver and kidney (as measured by either  $\text{O}^6$ - or 7-methylguanine levels) relative to the amount of methylation produced by non-deuterated [methyl- $^{14}\text{C}$ ]NEMA. In contrast, 7-methylation of esophageal DNA was ~2.6 times as great in animals receiving [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  compared to animals receiving non-deuterated [methyl- $^{14}\text{C}$ ]NEMA. The overall inter-organ shift in DNA methylation is best demonstrated by the liver/esophagus 7-methylguanine ratio (Table I). This value decreased from 109 (obtained using non-deuterated [methyl- $^{14}\text{C}$ ]NEMA) to 29 (using [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$ ). In lung, 7-methylguanine levels were comparable in animals treated with [methyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  and [methyl- $^{14}\text{C}$ ]NEMA.

The amount of 7-ethylguanine formed in hepatic DNA appeared to be slightly less in animals treated with [1-ethyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$  compared to those treated with undeuterated [1-ethyl- $^{14}\text{C}$ ]NEMA, slightly higher in kidney, and approximately double in lung. This resulted in a decrease in the liver/lung 7-ethylguanine ratio from 20, using undeuterated [1-ethyl- $^{14}\text{C}$ ]NEMA, to 8.2 using [1-ethyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$ . Ethylation of esophageal DNA, however, could not be detected after treatment with either [1-ethyl- $^{14}\text{C}$ ]NEMA or [1-ethyl- $^{14}\text{C}$ ]NEMA- $\text{d}_3$ .

To directly compare the ratio of methylation to ethylation by NEMA- $\text{d}_3$  and NEMA, the 7-methylguanine/7-ethylguanine ratio has been included in Table I. This ratio is slightly lower (by 17%) for liver using NEMA- $\text{d}_3$  and dramatically lower (by 40–50%) for kidney and lung, indicating that there is proportionately more ethylation (and/or less methylation) in these tissues for deuterated than for non-deuterated NEMA.

Table I. Comparison of DNA alkylation by NEMA and NEMA-d<sub>3</sub> in the organs of Fischer 344 rats<sup>a</sup>

	NEMA <sup>b</sup>		NEMA-d <sub>3</sub> <sup>c</sup>	
	[methyl- <sup>14</sup> C]	[1-ethyl- <sup>14</sup> C]	[methyl- <sup>14</sup> C]	[1-ethyl- <sup>14</sup> C]
Liver				
7-alkG	1014 ± 71	6.0 ± 0.4	690 ± 49	4.9 ± 0.2
O <sup>6</sup> -alkG	103 ± 10	3.5 ± 0.5	71 ± 7	2.4 ± 0.1
O <sup>6</sup> -alkG/7-alkG <sup>d</sup>	0.10 ± 0.01	0.58 ± 0.09	0.10 ± 0.01	0.49 ± 0.03
7-meG/7-etG		169		141
O <sup>6</sup> -meG/O <sup>6</sup> -etG		29		30
Kidney				
7-alkG	73 ± 3	0.4	48 ± 5	0.45 ± 0.03
O <sup>6</sup> -alkG	4.0 ± 0.5	n.d.	2.8 ± 0.4	0.14 ± 0
O <sup>6</sup> -alkG/7-alkG <sup>d</sup>	0.055 ± 0.007	—	0.058 ± 0.010	0.31 ± 0.02
7-meG/7-etG		183		107
O <sup>6</sup> -meG/O <sup>6</sup> -etG		—		20
Esophagus				
7-alkG	9.3	n.d.	24	n.d.
Lung				
7-alkG	3.8 ± 0.3	0.3	3.9 ± 0.1	0.6
O <sup>6</sup> -alkG	n.d.	n.d.	n.d.	0.08
O <sup>6</sup> -alkG/7-alkG	—	—	—	0.13
7-meG/7-etG		13		6.5
7-alkG ratios				
Liver/kidney	14	15	14	11
Liver/esophagus	109	—	29	—
Liver/lung	267	20	177	8.2

<sup>a</sup>Male Fischer 344 rats received a single i.p. dose (4.4 mg/kg) of [methyl-<sup>14</sup>C]NEMA, [1-ethyl-<sup>14</sup>C]NEMA, [methyl-<sup>14</sup>C]NEMA-d<sub>3</sub>, or [1-ethyl-<sup>14</sup>C]NEMA-d<sub>3</sub> and were killed 4 h later. Alkylated purines are expressed as  $\mu$ mol/mol guanine. Abbreviations: 7-alkG, 7-alkylguanine; O<sup>6</sup>-alkG, O<sup>6</sup>-alkylguanine; 7-meG, 7-methylguanine; 7-etG, 7-ethylguanine; O<sup>6</sup>-meG, O<sup>6</sup>-methylguanine; O<sup>6</sup>-etG, O<sup>6</sup>-ethylguanine; n.d., not detected.

<sup>b</sup>Data from von Hofe *et al.* (5), means  $\pm$  SD of three determinations for liver, two for methylation values in kidney and lung, and single analyses of pooled samples for ethylation in the latter two organs plus methylation in the esophagus.

<sup>c</sup>Means of three determinations  $\pm$  SD for liver alkylation and kidney methylation or two replicates for renal ethylation and lung methylation. Esophagus data and those for lung ethylation are from single analyses of pooled tissue DNA.

<sup>d</sup>Ratios of O<sup>6</sup>-alkG to 7-alkG values  $\pm$  SD.

For comparison with the *in vivo* alkylation data, the microsomal metabolism of the two nitrosamines was also studied. Kinetic constants for their conversion to the two  $\alpha$ -hydroxylation products, acetaldehyde and formaldehyde, by uninduced liver microsomes are summarized in Table II. As was found for the rates of *in vivo* clearance of NEMA (7), no significant change in  $K_m$  or  $V_{max}$  values could be observed on  $\beta$ -deuteration of the carcinogen.

## Discussion

The finding of Lijinsky *et al.* (2) that  $\beta$ -trideuteration of NEMA converts it from a liver-specific carcinogen to one with additional activity in esophagus offers an unusual opportunity to probe the mechanism of organotropism for a simple *N*-nitroso compound. We originally speculated that this shift toward the esophagus resulted from an isotopically-induced retardation of  $\beta$ -hydroxylation during the first pass through the liver following absorption from the gut of the orally-administered carcinogen, but this proved to be incorrect. Detailed pharmacokinetic studies failed to detect any difference between NEMA and NEMA-d<sub>3</sub> in bioavailability or any other pharmacokinetic parameter, nor was any of the  $\beta$ -hydroxylation product, *N*-nitrosomethyl(2-hydroxyethyl)amine, found in the blood at a detection limit corresponding to 0.3% of the dose (7). Also, the organotropic shift could not

Table II. Kinetic parameters for metabolism of NEMA and NEMA-d<sub>3</sub> by rat liver microsomes<sup>a</sup>

Metabolite	$K_m$ (mM)		$V_{max}$ (nmol/min/mg of protein)	
	NEMA	NEMA-d <sub>3</sub>	NEMA	NEMA-d <sub>3</sub>
Acetaldehyde	1.41 ± 0.29	1.48 ± 0.08	4.44 ± 1.22	4.61 ± 1.69
Formaldehyde	7.25 ± 5.46	5.02 ± 4.51	1.50 ± 0.98	1.49 ± 0.46

<sup>a</sup>Means  $\pm$  SD of six (NEMA) or four (NEMA-d<sub>3</sub>) determinations.

be ascribed to altered excretion of unchanged nitrosamine, as neither NEMA nor NEMA-d<sub>3</sub> could be found in the urine at a level of 0.03% of the dose (7).

Rather, the present results suggest that the observed organotropic shift is attributable to a different sort of metabolic switching. It has already been shown that  $\alpha$ -deuteration of simple nitrosamines such as NEMA (11) and *N*-nitrosodimethylamine (12,13) can retard metabolism at the deuterium-substituted position, and one might expect deuteration of the  $\beta$ -carbon to result in a similar retardation. Assuming that the ethyl group of NEMA would normally be oxidized in esophagus at both the  $\beta$ - and  $\alpha$ -carbons, retardation of  $\beta$ -attack by the presence of deuterium at that position could make  $\alpha$ -oxidation relatively more extensive by default. This would lead in turn to more extensive

alkylation of DNA at a given molar dose level and increased carcinogenic potency. As predicted from this hypothetical interpretation, the levels of guanine methylation in esophageal DNA were more than doubled in animals receiving NEMA-d<sub>3</sub> relative to those receiving NEMA, and the 7-methylguanine ratio for liver/esophagus decreased four-fold (from 109 to 29). Ethylation, however, still could not be detected in esophageal DNA using NEMA-d<sub>3</sub>. These results are consistent with the report that DNA methylation is of primary importance in the induction of esophageal neoplasms by methylalkylnitrosamines (4), and we conclude that the postulated metabolic switching mechanism offers a reasonable explanation for the relatively great carcinogenic potency of NEMA-d<sub>3</sub> in rat esophagus.

The fact that increased methylation is observed only in the esophagus suggests that other organs strike a different balance among the various possible competing pathways of NEMA metabolism. For example, the lower methylation (but not ethylation) of kidney DNA by NEMA-d<sub>3</sub> compared to NEMA suggests that one or more metabolic pathways in that organ are responsible for  $\beta$ -oxidizing NEMA to an alkylating agent capable of methylating (but not ethylating) DNA. Again,  $\beta$ -deuteration would inhibit the formation of this species, giving rise to diminished quantities of 7- and O<sup>6</sup>-methylguanine (Table I). As to the identity of such an alternate methylating agent, a plausible candidate has recently been suggested (14). Attachment of substituents that are easily displaced by nucleophiles at the  $\beta$ -position of NEMA is known to activate it toward intramolecular attack on the nitrosamino group to produce the 3-methyl-4,5-dihydro-1,2,3-oxadiazolium ion (15). This highly polar species is a direct-acting mutagen (16) and methylating agent (17) whose formation from NEMA via the net insertion of a sulfate ester function (14–20) or similar leaving group at the  $\beta$ -carbon can easily be envisioned. Formation of this intermediate should be hindered by the presence of deuterium at the  $\beta$ -position of NEMA, thereby reducing the level of DNA methylation. Ethylation, on the other hand, cannot occur by a  $\beta$ -oxidation mechanism and therefore should not be greatly affected by deuteration at the  $\beta$ -position. It is noteworthy that coadministration of ethanol with NEMA results in a shift in DNA adduct formation (5) similar to that observed for NEMA-d<sub>3</sub>, suggesting that ethanol may also preferentially inhibit reactions at the  $\beta$ -position of NEMA in kidney.

The similarity of the lung alkylation data for NEMA relative to NEMA-d<sub>3</sub> (Table I) suggests that the cytochrome P450 isozymes primarily responsible for NEMA metabolism in that organ are not greatly influenced by deuterium substitution at the  $\beta$ -carbon; here again, the result with deuteration mimics that seen previously on ethanol coadministration (5). As for the liver, the lower alkylation levels with NEMA-d<sub>3</sub> might have been expected to correlate with weaker carcinogenic effects; the fact that it was at least as active as NEMA in the chronic administration studies (2) may only reflect their similar positions on the high-dose plateau of the dose–response curve.

In conclusion, the present results show that the high incidence of esophageal tumors observed with NEMA-d<sub>3</sub> is paralleled by an inter-organ shift of DNA methylation from liver to esophagus. This is consistent with previous studies (4) suggesting that methylation of esophageal DNA is a critical determinant of that organ's susceptibility to carcinogenesis by nitrosamines. It is not yet clear how  $\beta$ -trideuteration of NEMA causes this inter-organ shift in DNA alkylation, though presumably decreased metabolism at the  $\beta$ -carbon (due to the increased strength of the C–D bond) must play a role. Pharmacokinetic differences between

NEMA and NEMA-d<sub>3</sub> have been ruled out (7), as have differences in microsomal metabolism, at least for the liver (Table II). This would support the hypothesis that in esophagus the inhibition of metabolism by deuterium at the  $\beta$ -position results in a switching toward increased hydroxylation at the  $\alpha$ -methylene carbon which subsequently results in more DNA methylation. The data further suggest that in organs where  $\beta$ -trideuteration of NEMA results in decreased DNA methylation (e.g. kidney), metabolism at the  $\beta$ -position of NEMA may result in a polar intermediate which is capable of methylating but not ethylating renal DNA. Further studies are required to determine if these hypotheses are correct or whether other mechanisms are responsible for the inter-organ shift in NEMA metabolism resulting from  $\beta$ -deuteration of this carcinogen.

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